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# Sample Preparation for Bioanalytical and Pharmaceutical Analysis

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## **Disciplines**

Chemistry

## **Comments**

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## Sample Preparation for Bioanalytical and Pharmaceutical Analysis

Biological and pharmaceutical samples represent formidable challenges in sample preparation that hold important consequences for bioanalysis and genotoxic impurity quantification. This Feature will emphasize significant advances toward the development of rapid, sensitive, and selective sample preparation methods.

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Image created by O'Reilly Science Art, LLC. Attributions for protein structures: Cofilin-1 (PDB ID 5HVX), Galectin-1 (PDB ID 4Y1V), and Glyceraldehyde-3-Phosphate Dehydrogenase (PDB ID 4Z0H).

A form of sample preparation prefaces virtually every analysis of a complex sample. Converting the sample into a format that is compatible with analytical instrumentation may be as straightforward as dilution or filtration or may involve multistep sample handling procedures. As one of the first stages in the analytical process, the selection of an appropriate sample preparation technique is critical for obtaining reliable downstream measurements and consequently warrants careful consideration.<sup>1</sup> Many conventional methods entail significant user intervention, are time-consuming, and exhaust large quantities of solvent and/or other consumables. Furthermore, the demand for improved method sensitivity and selectivity has begun to rapidly eclipse the capabilities of traditional sample preparation techniques.<sup>2</sup> This is particularly relevant for complex sample matrixes where highly efficient, more selective, and low-cost extraction/purification alternatives are becoming increasingly desirable.

Biological samples represent formidable sample preparation and analysis challenges due to the presence of interfering constituents within the sample matrix. Contaminants in these complex samples may clog sampling and extraction devices, nonspecifically interact with analytes, coextract with target compounds, and/or foul instrumentation through adsorption.<sup>3,4</sup> Adding to the difficulty, the sample matrix often contains only trace levels of analyte that require a form of preconcentration or purification in order to deliver a sufficient quantity of target compound to the analytical instrument.<sup>5</sup>

This Feature article will highlight a selection of sample preparation challenges that have significant impact in the life sciences and pharmaceutical industry. Important advances in these areas will be emphasized and can often be attributed to a fundamental understanding of the physicochemical properties of the analyte and sample matrix.

### ■ SAMPLE PREPARATION FOR NUCLEIC ACID ANALYSIS

Within biological systems, nucleic acids (NAs) are responsible for the storage and transfer of genetic information that is essential for organism function and development. Apart from their critical role in natural processes, the analysis of NAs has become indispensable for a multitude of scientific and medical disciplines including genomics, clinical diagnostics, food safety, and forensic analysis. Modern sequencing and detection techniques are capable of rapidly generating enormous amounts of information from high-quality NA. However, purification of DNA or RNA is often marked by laborious, time-consuming procedures and represents a significant bottleneck in the analytical workflow.

Since NA constituents represent an exceedingly small quantity of total cellular material, the isolation and preconcentration of NAs are requisite steps for most applications. Sensitive amplification techniques, such as polymerase chain reaction (PCR), are frequently employed to facilitate the detection of trace amounts of NA. However, enzyme-based amplification methods require the input of highly pure samples. Interferences that diminish amplification efficiency or inhibit PCR altogether may originate from the cell itself (e.g., proteins) or the surrounding environment (e.g., humic acids in soil) and must be removed prior to analysis. While the identity and abundance of interfering agents within a given sample often dictate the sample preparation method, NA extraction

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Table 1. Examples of Nucleic Acid Extraction Methods from Biological Samples

Extraction method	Nucleic acid/sample matrix	Downstream Application	Centrifuge/vacuum apparatus	Organic solvent/chaotrope	ref
Phenol-chloroform	DNA or RNA/tissue, plant, sediment	PCR	yes	yes	6–8
EA1 enzyme-based	DNA/buccal swab	On-chip PCR	not required	not required	16
Silica beads	<i>B. anthracis</i> DNA/whole blood	On-chip PCR	not required	yes	34
Polymeric ionic liquid	pDNA/bacterial cell lysate	qPCR	not required	not required	36, 38
Magnetic oligo(dT) beads	mRNA/3T3 cell lysate	RT-qPCR	not required	not required	43
Magnetic ionic liquid	pDNA/bacterial cell lysate	PCR	not required	not required	46, 47

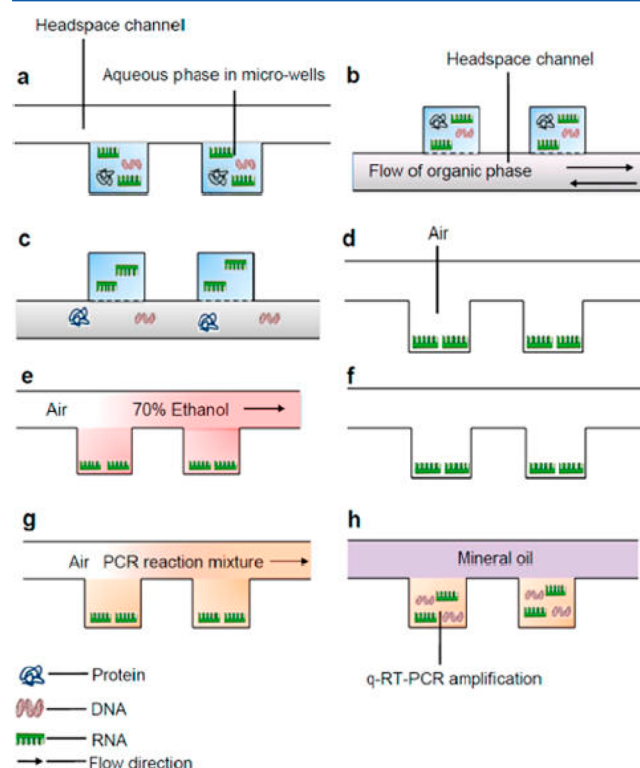
techniques generally possess common objectives that include cell lysis, separation of NAs from the bulk sample matrix, and reconstitution in a medium compatible with downstream experiments. Several examples of NA extraction methods are displayed in Table 1.

### ■ LIQUID PHASE NUCLEIC ACID EXTRACTION AND PURIFICATION TECHNIQUES

One of the earliest and most commonly used techniques for NA isolation relies on a liquid–liquid extraction (LLE) method involving a mixture of phenol and chloroform. In this classical approach, cells are lysed using detergents, chaotropes, or heat in order to release NAs into solution. Denatured proteins, lipids, and other cellular components are extracted into the phenol-chloroform layer while NAs remain in the aqueous phase. By controlling the pH of the system, the method may be applied for the selective isolation of DNA or RNA. Subsequent precipitation of the NAs is accomplished using ethanol or isopropanol, followed by reconstitution in a medium suitable for downstream analysis. Modification of cell lysis conditions/solvent composition has led the LLE technique to be widely adopted for NA isolation from tissues,<sup>6</sup> plant material,<sup>7</sup> and sediments.<sup>8</sup>

While phenol-chloroform extraction has been used for the purification of NAs from a variety of complex samples, the method is not without limitations. The multiple sample preparation steps required for this method are time-consuming, laborious, and severely reduce sample throughput. Furthermore, the large volumes of toxic organic solvent consumed in this approach have raised health and environmental concerns, leading many researchers to seek alternative solvent-based extraction methods. One approach to minimizing the use of organic solvent is to develop miniaturized NA extraction methods within microfluidic devices. Microfluidic systems are comprised of microchannels and chambers fixed in a chip through which small volumes of sample, solvent, and reagents are manipulated. Morales and co-workers designed a microfluidic device for the purification of NAs using a liquid extraction technique based on the differential partitioning of DNA and protein between an aqueous phase and an organic phase consisting of phenol, chloroform, and isoamyl alcohol.<sup>9</sup> To enhance the partitioning of protein to the organic phase, droplet-based flow was employed to maximize the interfacial area between the aqueous and organic phases. The microfluidic platform was applied for the purification of plasmid DNA (pDNA) from bacterial cell lysate with subsequent quantification by gel electrophoresis. In addition to minimizing the use of organic solvents, droplet-based DNA purification within microfluidic devices circumvents the need for manual sample handling procedures.<sup>10,11</sup> Zhang et al. later demonstrated the isolation and amplification of NAs from bacterial cell lysate with a microfluidic liquid phase purification system.<sup>12</sup> In their

approach, as shown in Figure 1, bacterial cell lysate was loaded into the microwells of the NA purification chip and a mixture of



**Figure 1.** Schematic of RNA purification using a microfluidic liquid phase extraction technique. Panels a–c show removal of protein and DNA from aqueous RNA within microwells. Drying and wash steps are depicted in panels d–f, enabling RT-qPCR as shown in panels g and h. (Adapted with permission from ref 12. Copyright 2013 American Chemical Society).

phenol, chloroform, and isoamyl alcohol was cycled back and forth over the wells to ensure partitioning of proteins to the organic phase. The organic phase was flushed to waste and residual organic solvent evaporated from the microwells by applying a vacuum, followed by washing of the dried NA with 70% ethanol. DNA and RNA extracted using this approach were sufficiently pure to serve as templates for on-chip real-time quantitative PCR (qPCR) and reverse transcription qPCR (RT-qPCR) analysis, respectively.

In some cases, organic solvent can be eliminated from NA sample preparation methods altogether. Enzyme-based methods have recently been described that enable rapid cell lysis and degradation of protein contaminants to liberate DNA for downstream analysis. The method relies on a proteinase from the thermophilic *Bacillus* sp. *Erebus antarctica* 1 (EA1) that extensively hydrolyzes cellular proteins, including nucleases,



when incubated at 75 °C.<sup>13</sup> Upon centrifugation of the resulting suspension to remove cell debris, DNA in the supernatant is suitable for PCR amplification. Since the enzyme-mediated extraction of DNA occurs within a closed sample tube, this technique is particularly useful for forensic analysis where minimizing the risk of sample contamination with exogenous DNA is of paramount importance. Lounsbury and co-workers investigated the extraction and PCR amplification of DNA from forensic samples, such as whole bloodstains and buccal swabs using the EA1 enzyme DNA extraction method.<sup>14</sup> The authors subsequently adapted the enzyme-based method to a poly(methyl methacrylate) (PMMA) microdevice and noted that the sample incubation time could be dramatically reduced (from 20 min to approximately 60 s) while still generating PCR-amplifiable DNA. The enzyme-based DNA method has also been employed in microdevices that integrate sample preparation, DNA amplification, and detection in single-use microfluidic chips.<sup>15,16</sup>

Recent efforts in the development of liquid phase NA extraction techniques not only seek to minimize organic solvent consumption and analysis time but also enhance the selectivity of the extraction media for NAs. Ionic liquids (ILs) constitute a growing class of solvents comprised of readily customizable organic cations and organic/inorganic anions with melting points at or below 100 °C. An attractive feature of ILs is the ability to synthetically install functional groups in the cation or anion component that facilitate specific interactions between the IL solvent and analytes. Wang and co-workers found that the 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM<sup>+</sup>][PF<sub>6</sub><sup>-</sup>]) IL was capable of extracting up to 99% of double-stranded DNA from aqueous solution using a LLE technique.<sup>17</sup> Using <sup>31</sup>P NMR to observe changes in the chemical shift of the DNA phosphate backbone, electrostatic interactions between the [BMIM<sup>+</sup>][PF<sub>6</sub><sup>-</sup>] IL and DNA were determined to be the driving force for DNA extraction. Further investigation of a variety of IL extraction solvents revealed that, in addition to electrostatic interactions, hydrogen bonding and hydrophobic interactions also played a role in the enhanced DNA extraction efficiencies.<sup>18</sup>

Buffer systems comprised of IL additives have proven useful for the extraction of genomic DNA from challenging sample matrixes including maize<sup>19</sup> and processed meats.<sup>20</sup> By stirring the biomass within a buffered solution of imidazolium or choline-based ILs at elevated temperature, NAs were released into solution and isolated by the sedimentation of cellular debris. Although DNA yields were reportedly lower than conventional extraction methods (e.g., surfactant-assisted extraction), the IL-based extraction provided DNA of sufficient purity for qPCR with a shorter overall sample preparation time.

## ■ SOLID PHASE EXTRACTION OF NUCLEIC ACIDS

Solid phase extraction (SPE) is a widely used sample preparation technique in analytical chemistry and has become increasingly popular for the isolation of NAs. A typical SPE procedure for NA purification involves cell lysis, binding of NAs to the sorbent material, a wash step to remove interfering agents, and elution of the NA in an appropriate buffer for downstream analysis. Numerous SPE sorbents have been reported for NA extraction including anion exchange resins,<sup>21</sup> alumina membranes,<sup>22</sup> and chitosan-modified particles.<sup>23</sup> However, silica-based substrates constitute the most widely applied materials for solid phase NA purification. Under

chaotropic conditions, DNA adsorbs to silica in a process thought to be facilitated by dehydration of DNA and the silica surface, hydrogen bonding interactions, and shielding of the negatively charged moieties on DNA and silica.<sup>24</sup> Lowering the solution pH results in superior DNA adsorption, likely due to neutralization of the weakly acidic silanol groups on the silica surface.<sup>25</sup> The adsorption of RNA to silica is also observed using similar conditions.<sup>26</sup> Importantly, the chaotrope-driven binding of NA to silica is reversible, where elution of DNA or RNA can be achieved using low ionic strength aqueous buffers.

Commercially available silica-based chemistries for NA purification exist in a variety of formats with spin columns and filters among the most common. After cell lysis and centrifugation of cellular debris, the aqueous supernatant rich in NAs is passed over the silica sorptive phase in the presence of a chaotrope using centrifugation or an applied vacuum. In many cases, enzymes such as proteinase K are added to the cell lysate to degrade proteins prior to the NA binding step. The sample may also be treated with ribonucleases (RNases) in order to enzymatically degrade RNA when selective isolation of DNA is desired. Although similar in SPE sorbent composition, comparison of DNA extraction performance for a number of silica spin columns from different manufacturers reveal a wide range of DNA yields and purities that are often sample matrix dependent.<sup>27,28</sup> Nonetheless, commercially available silica-based SPE methods have been successfully applied for NA isolation from complex matrixes including soil, food, and blood samples, with the capacity for semi or fully automated workflows.<sup>29</sup> However, automation of these centrifuge or vacuum-based macroscale SPE techniques requires sophisticated equipment that is cost-prohibitive to most users.

The translation of SPE technologies to microscale platforms represents an important advancement toward the development of inexpensive diagnostic systems that can readily function for rapid, on-site testing applications.<sup>30</sup> Some key advantages of miniaturizing NA sample preparation methods include reduced manual sample handling, decreased assay volumes, and shorter analysis times.<sup>31</sup> Initial efforts to incorporate silica-based extraction phases within microfluidic channels involved the fabrication of silica micropillars possessing high surface area for DNA capture.<sup>32</sup> In this approach, an aqueous DNA solution was flowed through the device in the presence of a chaotrope to bind NA to the micropillars. Following an ethanol wash, DNA could then be eluted using a Tris-EDTA buffer. A similar bind, wash, and elute protocol was followed to extract *Hind*III digested  $\lambda$ -phage DNA from aqueous solution using a microdevice loaded with silica beads.<sup>33</sup> Off-line PCR amplification experiments demonstrated sufficient purity of the NA extracted by the microdevice. A fully integrated microfluidic system was later developed by Easley and co-workers that incorporated the necessary on-chip processes for genetic analysis of whole blood samples.<sup>34</sup> Since the reagents used for silica-based DNA extraction act as PCR inhibitors (e.g., chaotropes and alcohols), the authors carefully isolated the SPE and PCR portions of the device.

In contrast to relatively mature silica-based DNA extraction methodologies, polymeric ionic liquid (PIL) substrates have recently been explored as tunable DNA extraction sorbents. Wang and co-workers prepared PIL microspheres using an imidazolium-based IL monomer and a *N,N'*-methylene bis-(acrylamide) cross-linker for the extraction of pDNA from bacterial cell lysate.<sup>35</sup> The DNA binding capacity of the PIL microspheres was nearly 200  $\mu\text{g mg}^{-1}$  with 80% of the DNA

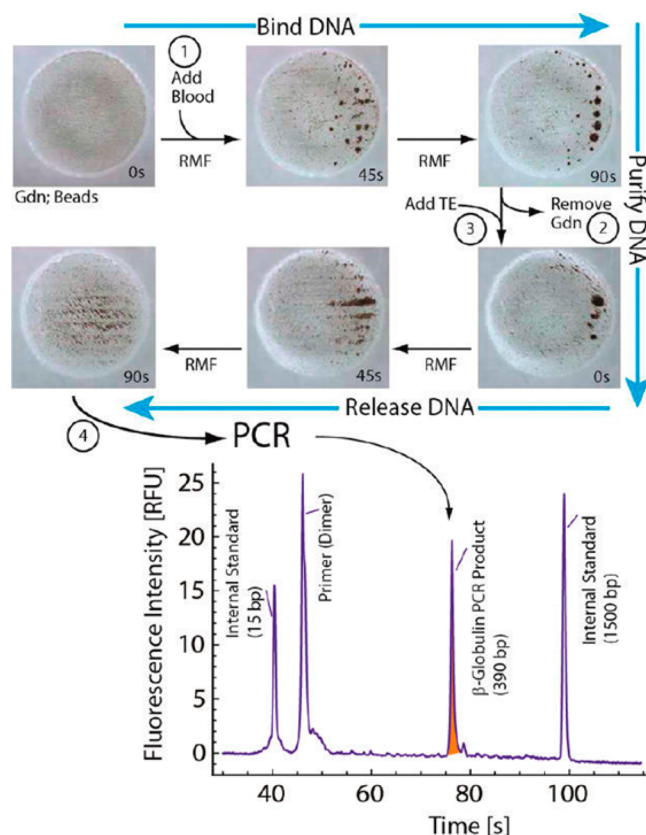
recovered when a sodium chloride desorption solution was employed. PILs have also been applied as sorbent coatings for the extraction of DNA using solid phase microextraction (SPME).<sup>36</sup> SPME, which was developed by Pawliszyn and co-workers in 1990,<sup>37</sup> is a technique in which analytes are extracted by exposing a thin fiber coated with a layer of sorbent to a sample solution. This technique combines sampling and sample preparation into a single step, which can significantly increase sample throughput while also decreasing the cost of the analysis due to the reusability of the SPME device. Using a PIL-based SPME device consisting of an imidazolium-based monomer and dicationic IL cross-linker, DNA was extracted from crude bacterial cell lysate and analyzed by qPCR.<sup>38</sup> In this approach, DNA extraction was found to proceed through an ion-exchange mechanism in which the negatively charged phosphate groups in DNA exchanged with the halide anions of the PIL sorbent coating. The SPME platform showed considerable promise for high-throughput NA analysis while circumventing the need for organic solvents and centrifugation.

### MAGNET-BASED SAMPLE PREPARATION FOR NUCLEIC ACIDS

Many LLE and SPE NA purification methods revolve around tedious, manual sample handling procedures and lengthy centrifugation steps that result in limited sample throughput. These challenges are unsustainable for high-throughput laboratories where NA analysis plays an essential role. The development of magnetoactive extraction media has provided a platform that is ideally suited to address the shortcomings of traditional NA sample preparation techniques. Magnet-based approaches utilize a magnetic extraction phase for the rapid enrichment and manipulation of NAs. In a process that bypasses centrifugation, extracted NAs can be easily isolated from the bulk sample matrix by the simple application of a magnetic field.

Magnetic beads and particles for DNA/RNA separations often consist of a magnetic core (e.g., iron oxide) encapsulated by various functional coatings that are capable of reversibly binding NAs.<sup>39</sup> When employed for the extraction of DNA from cell lysates, homogenized tissues, and other complex biological samples, the DNA-enriched sorbent can be magnetically controlled and separated from cellular debris and solid interferences. Using well-established silica-based binding chemistry, commercially available silica-coated paramagnetic particles extract DNA under chaotropic conditions.<sup>40</sup> Leslie and co-workers developed a method for DNA sample preparation and quantification using magnetic silica beads.<sup>41</sup> In this approach, human blood samples were lysed using guanidine hydrochloride and subsequently mixed with magnetic silica beads within a PMMA microwell. When a rotating magnetic field was applied, adsorption of human genomic DNA to the silica surface resulted in aggregation of the magnetic beads. The bead aggregation formed the basis for an optical detection and quantification method where a greater degree of aggregation indicated higher DNA concentration. As shown in Figure 2, release of the DNA from the magnetic beads with a low ionic strength buffer reversed the aggregation and yielded PCR-amplifiable template.

Functionalization of magnetic beads with single-stranded oligonucleotides represents a useful approach for the sequence specific enrichment of NAs. By selectively extracting a particular NA sequence, background signals produced by untargeted molecules in sensitive bioanalytical assays can be minimized.

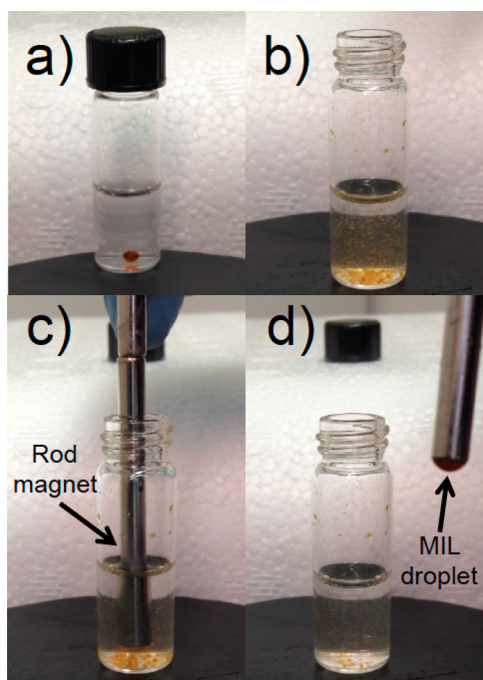


**Figure 2.** Extraction of DNA from whole blood using magnetic silica bead aggregation. DNA is released from the beads using Tris-EDTA, amplified using PCR, and the PCR products separated using electrophoresis. (Adapted with permission from ref 41. Copyright 2012 American Chemical Society).

These magnetic substrates are uniquely suited for the capture of polyadenylated mRNA via hybridization when appended with a polythymine nucleotide sequence (oligo(dT)). After cell lysis and target sequence hybridization, noncomplementary NA sequences can be removed by a washing step. Magnet-based sequence specific extraction has been leveraged for rapid mRNA extraction from blood,<sup>42</sup> single cell mRNA capture,<sup>43</sup> and mutation analysis.<sup>44</sup>

Very recently, magnetic ionic liquid (MIL) solvents were examined as DNA extraction media. MILs are a subclass of ILs that possess similar tunable physicochemical properties while also exhibiting susceptibility to magnetic fields.<sup>45</sup> Using a similar approach to that displayed in Figure 3, highly efficient extractions of salmon testes DNA from aqueous solution (~90% efficiency) were observed using microvolumes of tetrahaloferrate(III)-based MILs.<sup>46</sup> Furthermore, MILs were applied for the extraction of pDNA from crude bacterial cell lysate in a magnet-based method that was directly interfaced with PCR amplification.<sup>47</sup> The MIL-based approach was capable of yielding PCR-amplifiable pDNA without organic solvents or centrifugation in a process that required less than 2 min of sample preparation. When coupled with the DNA preservation capabilities of these magnetic solvents,<sup>48</sup> a workflow involving DNA extraction, storage, and subsequent analysis can be accomplished using PCR-compatible MILs.





**Figure 3.** Depiction of MIL-based extraction. (A) 20  $\mu\text{L}$  droplet of MIL in 2 mL of aqueous DNA solution. (B) Dispersion of MIL into fine droplets using agitation. (C and D) Retrieval of DNA-enriched MIL droplets using a 0.66 T rod magnet. (Adapted with permission from ref 45. Copyright 2016 Elsevier).

### ■ EXTRACTION AND PURIFICATION OF METABOLITES AND BIOMARKERS FROM BIOLOGICAL SAMPLES

Metabolomics is a field of research concerned with the comprehensive analysis of low molecular weight metabolites in biological systems. The analysis of such compounds promises to offer deeper insight into the mechanisms of disease and provide important biomarkers for diagnostic applications. Moreover, a greater understanding of the lifestyle and dietary factors that contribute to specific diseases can be gleaned from metabolic signatures.<sup>49</sup> One major challenge in metabolomics is the lack of approaches that are capable of identifying, detecting, and quantifying a broad range of metabolites that may span several orders of magnitude in their respective concentrations. Analytical instrumentation and/or hyphenated techniques including gas chromatography or liquid chromatography combined with mass spectrometry (GC–MS or LC–MS), nuclear magnetic resonance spectroscopy (NMR), and capillary electrophoresis (CE) are frequently employed in an attempt to fully characterize the metabolites within a given biological sample.<sup>49</sup> Although direct instrumental analysis minimizes the loss of analytes, interfering agents within the sample matrix (e.g., salt and macromolecules) can significantly affect the sensitivity and reproducibility of the analytical method. For example, excess salt content in the sample matrix may result in adduct formation and/or ion suppression during MS analysis.<sup>49,50</sup> In GC applications, the accumulation of non-volatile interferences in the inlet and at the head of the GC column can lead to active site formation, analyte degradation, and result in retention time shift and poor quantitation. To solve these issues, an appropriate sample preparation method must be judiciously selected and employed prior to

instrumental analysis, particularly when confronted with complex biological samples.

Protein precipitation is widely used in the processing of biological samples to concentrate proteins and purify them from various contaminants. It is also considered to be one of the fastest and simplest approaches for the removal of protein interferences from biological samples (e.g., serum and plasma). In practice, acetonitrile or methanol is injected into the sample to precipitate protein constituents followed by a centrifugation step to sediment the insoluble protein. This approach has been widely adopted for metabolomics profiling of plasma,<sup>51</sup> analysis of illicit drugs and their metabolites in oral fluid<sup>52</sup> and detection of vitamin D metabolites in plasma.<sup>53</sup> To further increase the throughput of the experiment, a fully automated protein precipitation procedure was developed by Watt et al. in which a 96-well plate and a robotic liquid handling system was employed for the sample preparation and analysis of more than 400 plasma samples per day.<sup>54</sup> Despite the advantages stated above, the sample may still contain a significant amount of soluble protein interferences due to the poor selectivity of this approach.<sup>55</sup> Moreover, coprecipitation of the analytes of interest can also diminish the recovery of the method.

Classical LLE is the most popular method for the extraction of metabolites from biological samples. When choosing the extraction solvent, a number of things need to be considered including toxicity, solubility, selectivity, chemical reactivity, and pH. Perchloric acid is one of the most suitable solvents for the extraction of polar, hydrophilic or basic compounds, such as primary metabolites. To ensure the reproducibility of metabolite analysis, quenching must be applied immediately after sample collection to prevent metabolite degradation or decomposition that can alter the original metabolite profile of the organism. One advantage of using perchloric acid as an extraction solvent is that it immediately quenches enzymatic reactions and denatures proteins, yielding a protein free extraction. However, this approach requires pH adjustment for each sample prior to NMR analysis to avoid peak shifts in the spectra.<sup>56</sup> Furthermore, the presence of perchlorate salts can make the method incompatible with chromatographic separations. For the extraction of moderately polar and nonpolar metabolites, organic solvents such as methanol or ethanol are often employed. Binary mixtures of solvents (e.g., mixture of methanol and water/acidified water) typically provide superior results when the extraction of a broader range of metabolites is desired.<sup>57</sup>

Although conventional LLE methods have been widely adopted for the isolation and preconcentration of metabolites in biological samples, a number of shortcomings still remain. LLE consumes relatively large volumes of organic solvents with expensive disposal requirements. During the LLE process, emulsions may form that prevent adequate phase separation and, consequently, lead to difficulties in quantitative recovery of the extracted analytes. Moreover, LLE often requires time-consuming and laborious sample handling procedures that are not readily automated without the use of expensive and sophisticated equipment. Advances in liquid phase extraction seek to address these issues with liquid–liquid microextraction (LLME) techniques, including single-drop microextraction (SDME), hollow fiber liquid phase microextraction (HF-LPME), and dispersive liquid–liquid microextraction (DLLME). When coupled to state-of-the-art analytical instrumentation, these methods often provide faster analysis times, higher sample throughput, lower solvent consumption,

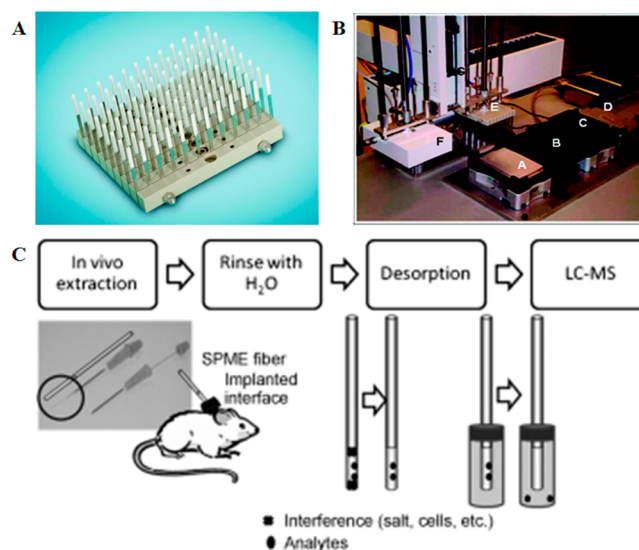
and even higher sensitivity. Though not the focus of this Feature, recent developments in LLME techniques for bioanalytical applications have been reviewed.<sup>55,58</sup> Because of their user-friendliness and cost-saving potential, it is expected that these techniques will continue to play an important role in the future of metabolite analysis.

SPE is a well-established method for sample cleanup and preconcentration of semivolatile/nonvolatile metabolites in biological samples at trace levels. This technique has many advantages including high recovery, effective preconcentration, and lower organic solvent consumption (compared to LLE) and is generally easy to automate.<sup>55</sup> Furthermore, SPE devices (e.g., columns, cartridges) may also function as a filter preventing suspended solids from contaminating or clogging instrumentation. This is particularly relevant for complex biological sample matrices for which a filtration step following protein precipitation is usually required.<sup>59</sup> In practice, the sample is introduced into the SPE device and analytes of interest partition between a solid extraction phase and a liquid phase. The SPE sorbent can be chosen to strongly retain interfering matrix components and thus remove them from the sample or to selectively retain analytes while matrix components pass through to waste. The latter approach is most commonly applied since analytes can then be preconcentrated by using small volumes of eluent. In order to select the appropriate SPE sorbent for the method, it is important to consider the physical and chemical properties of the target analytes and the sample matrix. Common SPE sorbents include chemically bonded silica with various functional groups (e.g., C8 and C18), carbon or ion-exchange materials, polymeric materials (e.g., cross-linked styrene-divinylbenzene), immunosorbents, molecularly imprinted polymers, restricted access materials, and monolithic sorbents.<sup>60</sup> SPE methods have also been configured for online sample preparation prior to an analytical separation in order to increase sample throughput and minimize tedious sample handling procedures.<sup>61</sup>

While useful in many cases, SPE suffers from disadvantages including time-consuming method development, the potential for irreversible analyte adsorption, and higher cost since as SPE cartridges are often designed for single use. Moreover, SPE typically requires organic solvents for elution and the poor batch-to-batch reproducibility with respect to the sorbent material can also be of concern.<sup>55</sup> To address these challenges, SPME has been employed as a rapid and cost-effective method for the extraction of metabolites with a broad range of properties. Analytes that are preconcentrated using SPME are rapidly desorbed using a high temperature GC inlet or HPLC-compatible organic solvent for GC or HPLC analysis, respectively. For the analysis of metabolites in biological samples, the choice of the SPME sorbent coating depends on the purpose of the study. Adsorptive coatings like divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) are widely applied for metabolomics fingerprinting and profiling studies since they are capable of extracting compounds comprising a broad range of volatilities and polarities.<sup>62</sup> When a more targeted analysis is desired, selective coatings such as polyacryl (PA) and Carbowax may be more appropriate choices.

SPME can be performed using two different extraction modes, namely, headspace and direct immersion. In the headspace extraction mode, the SPME fiber is exposed to the headspace above the sample matrix (e.g., fruit, plant, human

tissue, and urine). This sampling approach avoids direct contact of the fiber with the sample and therefore minimizes the risk of contaminating the extraction phase. However, this method is only suitable for extracting highly volatile metabolites since nonvolatile or low volatility compounds are not readily transferred to the headspace.<sup>62</sup> In direct immersion SPME, the fiber is immersed in the sample whereby analytes partition between the sample matrix and the sorbent coating. This extraction mode can improve the capture of high molecular weight and polar metabolites. However, the adsorption of macromolecules on the surface of the fiber represents a potential drawback of direct immersion SPME and may result in fouling of the fiber and poor reproducibility. In one approach reported by Mirnaghi and co-workers, a biocompatible C18-polyacrylonitrile sorbent was applied for an automated 96-blade SPME system (see Figures 4A and B). The sorbent coating



**Figure 4.** (A) Concept 96-blade SPME device coated with C18-polyacrylonitrile; (B) schematic demonstration of autosampler for 96-blade SPME device. (C) *In vivo* SPME sampling of mouse blood. (Adapted with permission from refs 63 (Copyright 2011 American Chemical Society) and 65 (Copyright 2011 Wiley)).

exhibited good extraction recovery, long-term reusability, and good reproducibility for the extraction of diazepam, lorazepam, oxazepam, and nordiazepam from human plasma samples.<sup>63</sup> Another form of direct immersion SPME has recently emerged in which biocompatible sorbent coatings are applied for sampling and extraction of metabolites *in vivo*. This method combines extraction and metabolism quenching in a single step, which can significantly prevent the oxidation or enzymatic degradation of metabolites after removal from their natural biological milieu.<sup>64</sup> A schematic of *in vivo* SPME for global metabolomics studies of blood/plasma is shown in Figure 4C. When coupled with LC–MS, hundreds of metabolites could be extracted with high sensitivity and precision comparable to traditional methods (i.e., ultrafiltration, plasma protein precipitation). Moreover, *in vivo* sampling allowed detection of short-lived metabolites including  $\beta$ -NAD, AMP, and glutathione, which could not be detected by other methods.<sup>65</sup> To date, *in vivo* SPME has been successfully applied to various biological systems including microorganisms, plants, animals, insects, and human emissions.<sup>66</sup>



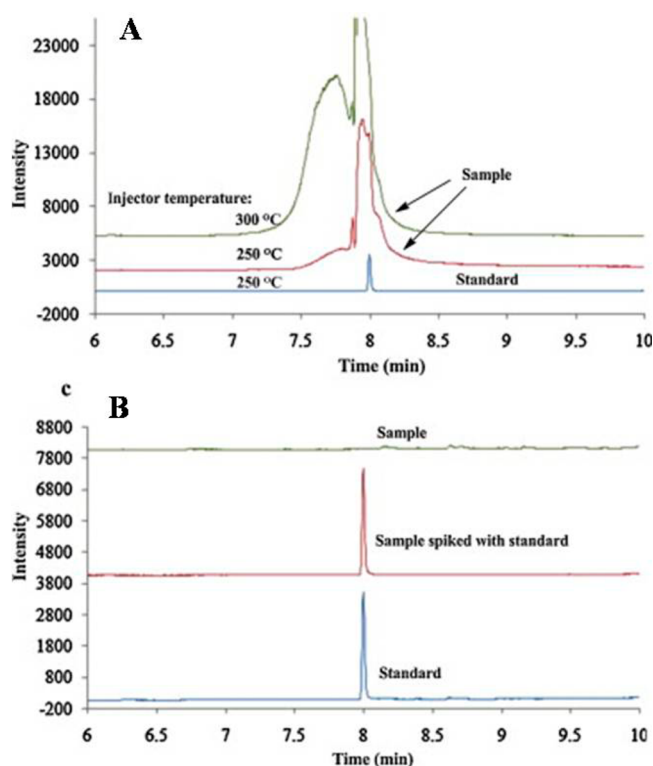
Stir bar sorptive extraction (SBSE) is another versatile sample preparation technique that involves the extraction and enrichment of metabolites from biological samples. This technique is based on the principle of sorptive extraction, where solutes are extracted by a polymer coating (e.g., PDMS) on a magnetic stirring rod. After extraction, the solutes can be desorbed using heat or a suitable solvent for GC or LC analysis. In comparison to SPME, a larger volume of extraction phase is used in SBSE, which can result in higher sensitivities for trace level analysis.<sup>67</sup> PDMS is the most widely used extraction phase for SBSE due to its broad temperature range and high stability toward various organic solvents. However, the limited solvation properties provided by PDMS render SBSE unsuitable for the extraction of polar compounds. Since metabolites within biological samples often possess high polarity, *in situ* derivatization prior to extraction has been used to yield higher sensitivity and improved chromatographic behavior.<sup>68</sup>

### ■ SAMPLE PREPARATION FOR GENOTOXIC IMPURITIES IN PHARMACEUTICAL ENTITIES

Trace level genotoxic impurities (GTIs) in pharmaceuticals are of increasing concern to both the pharmaceutical industry and regulatory agencies. The major source of GTIs is usually active pharmaceutical ingredient (API) manufacturing, which involves the use of genotoxic reagents, organic solvents, and catalysts.<sup>69</sup> GTIs are compounds that can possess unwanted toxicities, including genotoxicity and carcinogenicity. Stringent regulations were developed by the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to control the amount of GTIs tolerable in pharmaceutical entities.<sup>70,71</sup> Depending on the dose and duration of exposure, allowable daily intake values can be as low as 1.5  $\mu\text{g}/\text{person}/\text{day}$ , which is equivalent to low parts-per-million (ppm) or sub-ppm concentration ranges of GTIs in drug substances. Thus, monitoring the presence of various GTIs in drug substances is of great importance for the pharmaceutical industry and consumer health.

Direct injection of pharmaceutical samples to HPLC or GC is the most common approach for the determination and quantification of GTIs. Other approaches including chemical derivatization, matrix deactivation, and coordination ion spray MS have been reported to further enhance sensitivity.<sup>72</sup> However, since most APIs present in pharmaceutical samples exhibit low volatility, direct injection of the sample for GC analysis can result in contamination of the GC inlet and the head of the GC column. Moreover, in order to determine GTIs at low ppm levels, an extremely large quantity of API sample is usually required for direct injection analysis. This can result in serious band broadening of the main components (i.e., APIs) during chromatographic separation, increasing the complexity of the separation and peak integration. To address these issues, headspace GC (HS-GC) has been reported for the analysis of volatile GTIs.<sup>73</sup> This approach minimizes the amount of nonvolatile matrix components introduced to the GC by sampling only the gaseous components in a heated sample vial. Recently, ILs were used as a new class of diluents for the analysis of GTIs in small molecule drug substances by HS-GC. The low volatility and high thermal stability of ILs enables the method to be used at high HS-GC oven temperatures with minimal chromatographic background when compared to conventional HS-GC diluents such as dimethyl sulfoxide and dimethylacetamide. As a result, a significant improvement in the sensitivity for high boiling GTIs was achieved.<sup>74</sup>

Another approach for the analysis of GTIs involves API removal from the sample by extraction and purification methods, with LLE being the most common. Organic solvents such as methyl *tert*-butyl ether and *n*-hexane exhibited high selectivity for alkylating agents including dimethyl sulfate, alkyl mesylates, and alkyl besylates.<sup>75,76</sup> Yang and co-workers demonstrated that ethylmethanesulfonate (EMS) could be generated from the APIs in the GC inlet at high temperature via transesterification reaction, resulting in a considerable overestimation of EMS in APIs (see Figure 5A).<sup>77</sup> To solve this



**Figure 5.** (A) GC–MS chromatograms of standard and API samples obtained using the direct injection method (operated at different inlet temperatures). (B) GC–MS chromatograms of a standard, spike recovery sample, and sample after precipitation. (Adapted with permission from ref 77. Copyright 2015 Elsevier).

problem, the API sample was dissolved in an appropriate solvent (i.e., dichloromethane) followed by the addition of an antisolvent (i.e., *n*-hexane) to precipitate the matrix substance. EMS was extracted into the mixture of dichloromethane and *n*-hexane with little residual API and subsequently injected for GC–MS analysis. As shown in Figure 5B, interferences were removed following this sample pretreatment approach and the false positive for EMS was eliminated.

SPE has also been exploited as a preconcentration technique for the analysis of GTIs in APIs. An online HPLC–SPE–HPLC method was demonstrated by Yamamoto and co-workers for the selective determination of synthetic intermediates in APIs.<sup>78</sup> In this approach, the eluent from the first column was introduced via heart-cutting to a SPE column. Subsequently, analytes that retained on the SPE column were transferred to a second column for further separation. A linear range from 0.25 to 250 ppm was obtained.

SPME is also a powerful technique for the preconcentration of GTIs in drug substances. The PDMS/DVB and Carboxen/PDMS sorbent coatings were successfully applied for the

extraction/determination of GTIs (i.e., methyl and ethyl ester derivatives of select sulfonic acids, aziridine, and 2-chloroethylamine) in APIs.<sup>79,80</sup> Recently, the application of PILs as SPME sorbent coatings resulted in the parts-per-billion and parts-per-trillion ultratrace level quantification of GTIs and structurally alerting compounds (i.e., alkyl halides and aromatics).<sup>81</sup>

Since GTIs cannot be entirely eliminated from API production, purification must be performed until the concentrations of GTIs in APIs are lowered to acceptable levels. Conventional purification techniques include recrystallization, preparative HPLC, precipitation, and distillation. However, some of these methods are time-consuming and can result in low yields of API, which in turn increases the cost of the final product. Recently, selective API purification platforms including reactive scavengers,<sup>82</sup> organic solvent nanofiltration (OSN),<sup>83</sup> and molecularly imprinted polymers<sup>84,85</sup> have been developed. In one approach reported by Esteves and co-workers, a molecularly imprinted polymer in combination with OSN was employed for the successful removal of 99.7% of GTIs from the API while incurring a loss of just 8% API.<sup>85</sup> These emerging sample preparation methods will foreseeably provide valuable alternatives to conventional techniques in the future of rapid and selective GTI analysis.

## CONCLUSIONS AND OUTLOOK

Sample preparation is a key component of successful chemical analysis. Extraction, purification, and preconcentration techniques are particularly relevant for the quantification of trace analytes in complex sample matrixes. Some of the most demanding samples are derived from natural biological systems and consequently require innovative sample preparation methods to achieve selective or comprehensive analysis. The practical implementation of new sample preparation technologies will also be determined by sample throughput requirements, where the facilitation of laboratory automation can be expected to have substantial impact. Moreover, the continual development of highly efficient and selective extraction media is critical for meeting the necessary method detection limits enacted by regulatory agencies. The environmental outcomes of sample preparation must also continue to be considered. A number of advances have been made toward the miniaturization of extraction and purification systems that enable the handling of smaller sample and solvent volumes. These formats include microdevices, microextraction sorbents, and microextraction techniques that will likely continue to provide solutions where conventional macroscale sample preparation is limited.

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